Vreteno, a gonad-specific protein, is essential for germline development and primary piRNA biogenesis in Drosophila

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SUMMARY
In Drosophila, Piwi proteins associate with Piwi-interacting RNAs (piRNAs) and protect the germline genome by silencing mobile genetic elements. This defense system acts in germline and gonadal somatic tissue to preserve germline development. Genetic control for these silencing pathways varies greatly between tissues of the gonad. Here, we identified Vreteno (Vret), a novel gonad-specific protein essential for germline development. Vret is required for piRNA-based transposon regulation in both germline and somatic gonadal tissues. We show that Vret, which contains Tudor domains, associates physically with Piwi and Aubergine (Aub), stabilizing these proteins via a gonad-specific mechanism that is absent in other fly tissues. In the absence of vret, Piwi-bound piRNAs are lost without changes in piRNA precursor transcript production, supporting a role for Vret in primary piRNA biogenesis. In the germline, piRNAs can engage in an Aub- and Argonaute 3 (AGO3)-dependent amplification in the absence of Vret, suggesting that Vret function can distinguish between primary piRNAs loaded into Piwi-Aub complexes and piRNAs engaged in the amplification cycle. We propose that Vret plays an essential role in transposon regulation at an early stage of primary piRNA processing.

KEY WORDS: Germline stem cell, Soma, Transposon, Piwi, Aubergine, piRNAs, Tudor, Drosophila

INTRODUCTION
Propagation of all sexually reproducing organisms depends upon the faithful development and function of reproductive organs. In Drosophila, oogenesis requires the coordinated differentiation of two distinct cell lineages, the germline and the gonadal somatic cells, to produce an egg. The germarium, where oogenesis initiates, contains both germline and somatic stem cells. Asymmetric cell division of germline stem cells (GSCs) within the germarium generates both a stem cell and a differentiated daughter cell, the cystoblast, which gives rise to a sixteen-cell interconnected cyst (for a review, see Morrison and Spradling, 2008). One of the sixteen cells in the cyst differentiates into an egg and the remaining cells become nurse cells (King, 1970; Spradling, 1993). Somatic cell populations are intimately associated with germ cells during oogenesis: niche cells provide GSC maintenance signals and are tightly connected to GSCs via adhesion and gap junctions (Gilboa et al., 2003; Song et al., 2002; Xie and Spradling, 1998); inner sheath cells (ISCs) intermingle with the differentiating cystoblast and early dividing cysts to promote formation of the sixteen-cell cyst (Decotto and Spradling, 2005; Margolis and Spradling, 1995); follicle stem cells and their progeny, the follicle cells, surround each germline cyst as it buds off from the germarium and provide the maturing egg chamber with the positional cues needed for establishment of anterior-posterior and dorsal-ventral polarity of the embryo (Decotto and Spradling, 2005; Forbes et al., 1996; Margolis and Spradling, 1995; Roth and Schupbach, 1994; Zhang and Kalderon, 2001).

In addition to germline development, genomic integrity must be preserved to generate viable progeny. In Drosophila, transposable elements occupy nearly one third of the genome (Gubb et al., 1988) and mobilization of even one of almost 150 transposon classes found can lead to defects in gametogenesis and sterility (Bucheton et al., 1984; Kidwell, 1983; Pelisson, 1981; Rubin et al., 1982). Therefore, organisms have evolved small RNA-based defense systems to fight these elements (Malone and Hannon, 2009). In Drosophila, both germline and somatic cells of the ovary rely on Piwi proteins and their 23-29 nt Piwi-interacting RNAs (piRNAs) to combat transposon activity (Aravin et al., 2006; Girard et al., 2006; Houwing et al., 2007; Lau et al., 2006; Pelisson et al., 2007; Sarot et al., 2004; Vogin et al., 2006). All three Drosophila Piwi proteins, Piwi, Aubergine (Aub) and Argonaute 3 (AGO3), are expressed in germline cells, whereas Piwi is also expressed in somatic gonadal cells. Interestingly, mutations in all known piRNA pathway components lead to oocyte and embryonic patterning defects and, ultimately, to sterility, believed to be an indirect consequence of transposon-induced genomic instability and activation of a DNA double-strand break checkpoint (Klattenhoff et al., 2007; Theurkauf et al., 2006).
In contrast to other small RNAs, such as microRNAs and siRNAs, which are produced from double-stranded RNA precursors, piRNAs are derived from single-stranded RNA precursors, independently of the endonuclease Dicer (Vagin et al., 2006). piRNA precursors originate from either active transposon transcripts or discrete genomic loci known as ‘piRNA clusters’ (Brennecke et al., 2007). In Drosophila, piRNA clusters provide the primary source of antisense transposon transcripts, whereas active transposons predominantly provide sense transcripts (Brennecke et al., 2007; Gunawardane et al., 2007). piRNAs associated with Piwi and Aub are mostly derived from piRNA clusters, mapping complementary to active transposons, whereas AGO3-bound piRNAs appear to be derived from the transposon itself (Brennecke et al., 2007). This relationship and a 10 nt overlap observed between sense and antisense piRNA pairs led to a model of piRNA amplification termed ‘ping-pong’, in which 5’ ends of new piRNAs are generated through cleavage by the Piwi proteins themselves (Brennecke et al., 2007; Gunawardane et al., 2007). In the Drosophila ovary, piRNA ‘ping-pong’ is restricted to germline cells in which Piwi, Aub and AGO3 are present, although Piwi appears to be mostly dispensable for ‘ping-pong’ amplification (Malone et al., 2009). In gonadal somatic cells, in which only Piwi is expressed, an alternative pathway functions. Here, single-stranded piRNA clusters or gene transcripts are processed to produce ‘primary’ piRNAs that are directly loaded into Piwi, targeting active transposons or endogenous genes (Li et al., 2009; Malone et al., 2009; Saito et al., 2009). The overlapping genetic requirements for Piwi in the germline and ovarian somatic cells suggest that Piwi may also engage primary piRNAs in the germline. Like Piwi, the germline-specific Aub engages piRNAs complementary to transposons, but has not been directly linked to primary piRNAs. Therefore, the precise relationship between primary piRNAs and ‘ping-pong’ in the germline remains largely unknown.

The restriction of piRNA production and transposon control in gonadal tissues raises the question of how the piRNA biogenesis machinery has evolved specifically in the gonad. Here, we have identified Vreteno (Vret), a gonad-specific, Tudor domain-containing protein that functions specifically in the germline and somatic gonadal tissues during oogenesis. We show that Vret broadly regulates transposon levels and has an essential role in primary piRNA biogenesis, leaving ‘ping-pong’ amplification intact.

**MATERIALS AND METHODS**

*Drosophila* stocks

Oregon R and w1118 flies served as controls. vret48-60 and vret148-15 were recovered from an ethyl methanesulfonate (EMS) mutagenesis screen and vret21, vret22, vret70, vret29, vret67 and vret68 by non-complementation of vret21-15. Gal4 drivers used were: c587-Gal4 (Xie and Spradling, 1998); nos-Gal4-VP16 (Van Dorn et al., 1998); traffic-jam-Gal4 (Kyoto Stock Center); oua-Gal4 (Rorth, 1998); and apterous-Gal4 (from J. Treisman, NYU School of Medicine, NY, USA), gypsy-lacZ was a gift from A. Bucheton (CNRS, Montpellier, France); piwi1 and piwi2 from H. Lin (Cox et al., 1998); aubh102 and aubh102 (from T. Schupbach, Princeton University, NJ, USA) and UAS-aub-gfp from P. Macdonald (University of Texas, TX, USA). All other stocks were from the Bloomington *Drosophila* Stock Center.

Identification, mapping and molecular cloning of vret

vret was mapped by male mitotic recombination between P15010 and P16672, a 23 kb region uncovered by the deficiency Df(3R)Exel6192 (Bloomington *Drosophila* Stock Center). Single-nucleotide polymorphism (SNP) meiotic mapping between the recombinant line P15010, vret148 and P16672 yielded a polymorphism in the vret gene that identified the vret148 mutation.

**Immunofluorescence**

Adult ovaries were fixed and immunostained according to standard protocols. Wing imaginal discs immunostaining was performed as described (Roiqnt et al., 2006). Imaging was performed on a Zeiss Meta 510 LSM confocal microscope. All samples were stained and imaged under identical conditions.

**Vret antibody production and antibody reagents**

Glutathione-s-transferase-vret cDNA (2-367 amino acids) was isolated in inclusion bodies for production of rabbit polyclonal anti-Vret (Covance). Other antibodies used were: rabbit anti-Vasa (Lehmann laboratory) at 1:500; mouse 1B1 monoclonal supernatant (adducin-like) (Zaccaci and Lipshitz, 1996) at 1:20 and mouse anti-FasIII supernatant (7G10) at 1:10 (both from Developmental Studies Hyridoboma Bank); rabbit anti-Orb (Navarro et al., 2004) at 1:500; mouse anti-Myc Alexa555 conjugated-clone4A6 (Upstate) at 1:250; mouse anti-Myc 9E10 (AbCam) at 1:100; rabbit anti-cleaved Caspase-3 (Asp175) (Cell Signaling Technology) at 1:100; chicken anti-GFP (AVES) at 1:500; rabbit anti-GFP (Invitrogen) at 1:1000; mouse anti-β-gal (Promega) at 1:1000; rabbit anti-Piwi at 1:5000, rabbit anti-Aub at 1:1000 and rabbit anti-AO3 at 1:1000 (all three antibodies were provided by G. Hannon) (Brennecke et al., 2007); rabbit anti-Arm (a gift from W. Theurkauf) (Cook et al., 2004) at 1:10,000; mouse anti-α-tubulin (Sigma) at 1:50,000; mouse anti-β-tubulin (Sigma) at 1:2000; mouse anti-HA (Covance) at 1:200; mouse anti-Fibrillarin (EnCor Biotechnology) at 1:500; and DAPI (Roche) at 1:500 to visualize DNA. Alexa 488-conjugated Phalloidin (Molecular Probes) was used at 1:500. Secondary antibodies coupled to Alexa 488, Cy3 or Cy5 (Jackson Immunoresearch Laboratories) were used at 1:500.

**Clonal analysis**

vret germline clones were generated using the FLP/DFS (Flippase/Dominant Female Sterile) (Chou et al., 1993) or the FLP/GPL-marked clone (Xu and Rubin, 1993) systems. For FLP/DFS clones, second (L2) and third (L3) instar larvae were heat-shocked at 37°C for 2 hours on two consecutive days and fp122; FRT82B, vret48-60/FRT82B, ovwi2 adult females were fatened on yeast for 3 days for daily individual egg counts. For GFP-marked clones, L2 and L3 or 1- to 3-day-old adult flies of the genotype fp122; FRT82B vret48-60/FRT82B, nlsGFP were heat-shocked at 37°C for 2 hours on two consecutive days. Adult females were dissected 7 days after eclosion (when heat shock was carried out at larval stages) or 5 to 10 days after heat shock (when heat shock was carried out on 1- to 2-day-old adults).

**Generation of transgenic flies**

Full-length vret coding sequence from expressed sequence tag (EST) LD38352 [*Drosophila* Genomics Resource Center (DGRC)] and 5x-myc were amplified by PCR separately and subcloned into pGEM-7Zf (Promega) for sequencing. The vret-myc insert was then cloned into pUASp (Rorth, 1998). Full-length piwi coding sequence from EST RE21038 (DGRC) was amplified by PCR, cloned and recombined into the pPHW vector. Both pUASp-vret-myc and pUASp-HA-piwi transgenes were introduced into the *Drosophila* genome using standard P-element-mediated transformation techniques (Rubin and Spradling, 1982). Transgene functionality was verified by complementation of sterility phenotype in the respective mutant backgrounds.

**Microarray analysis**

Microarray analysis was performed in biological duplicates using total RNA extracted from *Drosophila* ovaries. The Affymetrix 3’-IVT Express Kit labeling protocol was applied followed by standardized hybridization and preassaying protocols using Affymetrix *Drosophila* 2.0 arrays. Transposable elements were identified on the arrays and their expression was analyzed in vret48-60/vret148, piwi1/piwi2 and aubh102/aubh102 ovaries after probe level summarization of the array intensities using a robust
multichip average (RNA-seq) algorithm. Each mutant was baseline-normalized to its corresponding heterozygote. Fifty-five significantly modulated probe set IDs corresponding to 52 unique transposable elements were identified in at least one of the three mutants analyzed, based on the statistical difference (t-test, P<0.05, at alpha level, no multiple testing corrections applied) between homozygotes and heterozygotes for each genotypic combination, combined with the minimum fold-change threshold (1.33, i.e. 33% change). All normalizations, statistical analyses, visualizations of hierarchical clustering results and Venn diagrams were performed in the Agilent GeneSpring GX11.5 platform. The array data is accessible from the NCBI Gene Expression Omnibus (GEO) public repository under accession number GSE30360.

**Immunoprecipitation and western blot analysis**

Ovaries were homogenized in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP40) and the supernatant was incubated with anti-Myc tag agarose conjugate (Millipore) for 2 hours at 4°C and then washed in NP-40 lysis buffer. Samples were run on NuPAGE 4-12% Bis-Tris gradient gels (Invitrogen). Rabbit anti-Vret was used at 1:2000, mouse anti-β-Tubulin at 1:10,000, mouse anti-α-Tubulin at 1:50,000, rabbit anti-Pwi at 1:5000, rabbit anti-Aub at 1:1000, rabbit anti-AGO3 at 1:1000, rabbit anti-Arm at 1:10,000, rabbit anti-Vasa at 1:20,000, mouse anti-Myc at 1:1000, mouse anti-Fibrillarin at 1:500 and rabbit anti-Orb at 1:1000. HRP-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:2000. An ECL-Western Blotting Detection Kit (Amersham) was used for visualization of horseradish peroxidase (HRP).

**Subcellular fractionation**

Ovaries were homogenized in hypotonic lysis buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT) by 10-20 strokes of a glass Dounce homogenizer. The cell suspension obtained was incubated on ice (homogenate fraction), centrifuged for 10 minutes at 3,000 rpm, 4°C in a microfuge chamber (Fig. 1E), and occasionally formed disorganized egg chambers (Fig. 1D; see Fig. S2B in the supplementary material). These mutants, follicle cells often failed to encapsulate egg chambers (Fig. 1E), and occasionally formed disorganized multicellular layers (Fig. 1G). Finally, vret2 and vret48-15 produced ventralized eggs but showed no defects in somatic gonadal cell patterning, germ cell differentiation or oocyte specification (see Table S1 in the supplementary material).

**Small RNA cloning, sequencing and analysis**

Small RNAs were purified, cloned and sequenced as previously described (Brennecke et al., 2007). In brief, 18-29 nt small RNAs were size-selected on a 15% polyacrylamide vertical gel, cloned and sequenced on the Illumina Genome Analyzer II platform. Small RNA sequence reads were clipped of their 3’ linker sequence and identical sequences were collapsed. Reads were mapped, allowing for zero mismatches, against the *Drosophila melanogaster* genome release 5.0. Only reads mapping to the genome, excluding unassembled heterochromatin, were used for further analysis. Reads were normalized to the total number of 20-22 nt endogenous small interfering RNAs (endo-siRNAs) derived from all 3’ UTR overlapping gene transcripts, as well as the esi-1 and esi-2 endo-siRNA clusters (Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008), as previously described (Malone et al., 2009). When mapping reads to the genome, no mismatches were allowed. When mapping to transposable element consensus sequences, up to three mismatches were allowed. When calculating ‘ping-pong’ signal (as described in Brennecke et al., 2008), piRNAs were mapped allowing for three mismatches. Small RNA libraries are deposited at Gene Expression Omnibus (accession number GSE30088, data sets GSM474629 and GSM474630).

**RESULTS**

**vreteno is required for germline and ovarian soma development**

CG4771 (hereby known as vreteno, the Bulgarian word for ‘wool-spinning spindle’, referring to its eggshell phenotype) was identified in a screen for maternal-effect mutations causing defects in oocyte polarity [see Materials and methods in Staeva-Vieira (E. Staeva-Vieira, PhD thesis, New York University, 2003)]. Point mutations were identified in the vret coding sequence for all eight alleles (Fig. 1A; see Tables S1, S2 in the supplementary material). Both female and male vret mutants are sterile. Females mutant for strong vret alleles lay no eggs, whereas mutants for weaker alleles produce ventralized egg chambers: the chorionic appendages, a marker for dorsal fate, were either fused or failed to form completely, a phenotype referred to as ‘spindle’ (see Table S1 in the supplementary material). Fertility was rescued in vret mutant females and males by a single copy of vret using a UASp-vret-myc transgene driven by the ubiquitously expressed actin5C-Gal4 driver (see Table S3 in the supplementary material).

In vret mutant ovaries, both germline and somatic gonadal cell development is affected. In the strongest vret alleles (vret48-60, vret53, vret48), the germarium was filled with germ cells that maintained a round spectrosome, suggesting failure of GSCs to differentiate (Fig. 1B,D). Moreover, ISC failed to associate with germ cells (Fig. 1D; see Fig. S2B in the supplementary material). Weaker vret mutants (vret39, vret15, vret48) progressed to later stages of oogenesis but egg chambers were defective with abnormal nurse cell numbers and improper oocyte positioning (Fig. 1C,E,F). In these mutants, follicle cells often failed to encapsulate egg chambers (Fig. 1E), and occasionally formed disorganized multicellular layers (Fig. 1G). Finally, vret2 and vret48-15 produced ventralized eggs but showed no defects in somatic gonadal cell patterning, germ cell differentiation or oocyte specification (see Table S1 in the supplementary material).

**Vret is a novel Tudor protein**

Vret contains two C-terminal Tudor domains (Fig. 1A), conserved motifs composed of four β strands forming an aromatic cage known to recognize and bind symmetrically dimethylated arginine residues (sDMA) (Liu et al., 2010; Maurer-Stroh et al., 2003). Alignment of the two Tudor domains in Vret [amino acids 376 to 422 (Vret tud1) and 581 to 626 (Vret tud2)] with other Tudor protein (tud11) (see Fig. S1A-B in the supplementary material). Fertility was rescued in vret using a actin5C-Gal4 driver (see Fig. S3A in the supplementary material).

Vret tud1 has three of the four aromatic residues, and Vret tud2 has only two. Three of the four Vret mis-sense mutations (vret59, vret87 and vret48-15), respectively, allowing a direct comparison of the relative role of the two domains. This glycine is highly conserved among Tudor domains and is likely to be important for holding the extended Tudor domain in a rigid structure (Liu et al., 2010). Interestingly, a glycine to glutamic acid change in Vret tud1 (vret59) exhibits a stronger phenotype than the same mutation in...
Vret2 (vret148-15). In particular, both point mutations in the Vret2 domain (vret49 and vret148-15) do not affect somatic gonadal development, suggesting a qualitatively different role of the two domains in Vret function. A fourth mis-sense mutation (vret49) is located N-terminal to the two Tudor domains and identifies an additional region critical for Vret function.

**Vret expression is gonad-specific and is required in both germline and soma for fertility**

Antibodies directed against amino acids 2-367 of the Vret protein detected a discrete, 80 kDa band in extracts of wild-type ovaries and testes (Fig. 1H). Vret expression was undetectable in fly carcass, in which gonads are absent, indicating that Vret is expressed in gonadal tissue (Fig. 1H). Full-length Vret protein is detected in both germline and soma for fertility (Fig. 1H). Vret expression is gonad-specific and is required autonomously in the germline for oocyte polarity (Fig. 2A,B; see Table S2 in the supplementary material). Removal of Vret from somatic cells had no effect on oocyte specification or localization, or the morphology of the follicle cell epithelium (Fig. 2B). However, clone size and cell-lineage specificity might have prevented us from detecting a soma-specific phenotype. We therefore expressed a UAS-p-vret-myc transgene under the control of tissue-specific Gal4 drivers. As expected, no rescue of the GSC differentiation phenotype was observed when Vret was expressed under the control of germline specific drivers, such as nos-Gal4-VP16 and ou6-Gal4 (see Table S3 in the supplementary material). By contrast, somatic drivers, including c587-Gal4 and traffic jam-Gal4, which drives expression in the germarial somatic cells (cap, ISCs and follicle precursor cells), and traffic jam-Gal4, which in addition drives expression in all follicle cells, rescued vret germline differentiation defects and somatic defects (see Table S3 in the supplementary material; compare Fig. 2C with 2D). Interestingly, bab1-Gal4, which expresses exclusively in terminal filament and cap cells, failed to rescue (see Table S3 in the supplementary material). Consistent with a role for Vret in early somatic cell lineages, we found that ISCs and accompanying somatic cells, which normally intermingle with germ cells, died in vret mutants as revealed by cleaved Caspase-3 expression (compare Fig. 2E with 2F). Together these results suggest that the failure in germline differentiation observed in the absence of vret is due to defects in the association between germ cells and their somatic neighbors.

**Fig. 1. vret is a gonad-specific, Tudor-domain protein required for germline differentiation and ovarian soma organization.** (A) vret encodes a protein of 691 amino acids that includes two Tudor domains in the C terminus. vret stop codon mutations are indicated above and point mutations below the diagram of the protein. (B) In vret148-60 heterozygotes, as in wild type, two to three GSCs with round spermatocytes are located at the anterior tip of the germarium (arrows), adjacent to the somatic niche, which is composed of terminal filament, cap cells and ISCs (arrowheads). A cystoblast (CB) and a differentiating cyst (dashed line) are indicated. The germ line is labeled with Vasa and somatic cell membranes and spectrosomes with 1B1. 1B1 staining in the GSC and CB marks single spermatocytes whereas in differentiating cysts it stains the fusome that connects the germ cells. (C) vret39 heterozygote shows a normal egg chamber with an oocyte specified at the posterior, labeled by Orb, and surrounded by a somatic follicle cell epithelial layer. (D) Germarium filled with single, undifferentiated germ cells in vret148-60 ovaries. (E) Defective egg chambers with mislocalized oocytes and incomplete encapsulation by follicle cell layers in vret39, a weaker vret allele. (F) vret39 mutant exhibits an abnormal number of nurse cells and a mislocalized oocyte. (G) Multilayered follicle cells partially envelope nurse cells in vret148-60 mutants. (H) Vret protein is detected in an immunoblot of wild-type adult ovary and testis lysates. β-Tubulin was used as loading control. (I) Vret antibody shows specificity in ovarian lysates of vret mutant alleles. Vret full-length protein (around 80 kDa) is not detectable in vret148-60. A weak but specific band of smaller molecular weight is detected in vret148-60, consistent with molecular data. A lower, non-specific band is observed in all samples (arrow), α-Tubulin was used as loading control.
Vret function in primary piRNA regulation

somatic support cells. Thus, Vret exhibits different tissue requirements: expression of Vret in the somatic gonad is required for germ cell differentiation, somatic gonadal cell survival and morphology, whereas Vret expression in the germline is required for oocyte and embryo polarity. Only expressing vret ubiquitously using actin5c-Gal4 rescued oogenesis to fertility (see Table S3 in the supplementary material).

Vret is required for transposon silencing in germline and somatic ovarian cells

Our analysis points to striking parallels between Vret and genes affecting the *Drosophila* piRNA pathway. First, Vret contains two Tudor domains, recently shown to associate with Piwi proteins (Liu et al., 2010; Nishida et al., 2009; Reuter et al., 2009; Vagin et al., 2009; Wang et al., 2009). Second, mutations in the two germline-specific *Drosophila* Piwi proteins Aub and AGO3 show oocyte polarity defects similar to those observed in vret germline clones (Li et al., 2009; Wilson et al., 1996). Finally, we found that mutations in both *flamenco* (*flam*), a piRNA cluster expressed exclusively in the ovarian soma, and *piwi* exhibited phenotypes similar to those observed in vret mutants, including defects in germ cell differentiation, somatic cell survival and follicle cell organization (Fig. 3A-F). We therefore investigated whether vret has a role in regulating transposable element activity. We analyzed the expression of the retroelement *gypsy* (Prud’homme et al., 1995), which is active in the somatic gonad and is regulated by piRNAs of the *flam* cluster, using a *gypsy-lacZ* transgenic strain (Sarot et al., 2004). While little β-galactosidase activity was observed in ovarian somatic cells of an otherwise vret heterozygous background (Fig. 3G), *gypsy-lacZ* accumulated significantly and specifically in the somatic epithelium of vret mutant ovaries (Fig. 3H). ZAM and *Idefix*, two other transposons regulated via the somatic Piwi/piRNA pathway (Desset et al., 2003), were also de-repressed in vret mutant ovaries as assayed by qPCR (data not shown).

We next tested whether vret was involved in global transposon regulation by performing microarray analysis. We found that most transposons contained in the *Drosophila* Genome 2.0 Array (Affymetrix), including those expressed specifically in the germline or somatic tissues of the ovary, were significantly de-repressed in vret homozygous mutants compared with heterozygotes (Fig. 3J; see Table S4 in the supplementary material). We also compared vret mutant ovaries with those of *piwi* and *aub* mutants and found numerous transposons similarly regulated in the three mutants (Fig. 3J; see Table S4 in the supplementary material). Piwi, like Vret, acts in both germline and somatic tissues of the gonad. Thus, similar elements were de-repressed in vret and *piwi* mutant ovaries, including *gypsy5*, *gtw1n*, *tabor* and ZAM, elements known to be regulated specifically in somatic cells (Fig. 3J; see Table S4 in the supplementary material). Furthermore, elements highly de-repressed in *aub* mutants were similarly de-repressed in vret mutants (Fig. 3J). Our results are consistent with a role for Vret in transposon regulation in both germline and somatic tissues of the *Drosophila* gonad.
Piwi proteins localization and accumulation depend on Vret

In wild type, Aub and AGO3 are expressed exclusively in germline cells and localize to a perinuclear structure known as ‘nuage’ in nurse cells (Harris and Macdonald, 2001; Li et al., 2009; Lim and Kai, 2007). Piwi, expressed in both germline and somatic cells of the ovary, is predominantly nuclear (Brennecke et al., 2007; Cox et al., 2000). We therefore investigated whether the localization and accumulation of Piwi proteins were affected in vret mutants. We compared mutant and wild-type expression within the same tissue by removing vret specifically from germline and/or soma by clonal analysis (see Materials and methods). The nuclear localization and protein accumulation of Piwi was almost entirely abolished in vret germline and somatic mutant clones throughout oogenesis (Fig. 4A¿B); see Fig. S3A in the supplementary material). Aub expression was severely reduced (Fig. 4C¿D) and nuage localization was affected in mutant germline cells (see Fig. S3B in the supplementary material). By contrast, no significant change in AGO3 expression was observed (Fig. 4D¿E) although the intracellular localization of AGO3 appeared punctate (see Fig. S3C in the supplementary material).

Consistent with the clonal analysis, Piwi and Aub protein levels were reduced in western blots whereas AGO3 protein remained at wild-type levels (Fig. 4E). Protein expression of two other piRNA pathway components, Armitage (Armi) and Vasa, were unaffected by loss of vret (Fig. 4E). Together, these results demonstrate that vret is required specifically for proper localization and accumulation of Piwi and Aub protein.

Vret associates with Piwi proteins in the ovary

To determine whether Vret affects Piwi and Aub at the transcriptional level, we analyzed tagged piwi and aub transgenes under the control of the heterologous UASp promoter,
driven by nos-Gal4-VP16 to achieve germline expression. Transgenic Piwi and Aub proteins, revealed by Hemagglutinin (HA) and GFP staining, respectively, as well as endogenous protein levels were severely diminished in vret mutants (Fig. 5A-D), demonstrating that Vret is not required for piwi and aub transcription but is somehow involved in post-transcriptional stability of these proteins.

To assess whether Vret is in a complex with Piwi or Aub we immunoprecipitated Vret from ovaries and probed lysates with antibodies against piRNA pathway components. We found that Vret specifically associates with Piwi and Aub (Fig. 5E). Vret also interacts with Armi and weakly with AGO3 (Fig. 5E). Although we observed a specific interaction between Vret and the piRNA components tested, it is unclear whether they are part of the same or separate complexes.

Piwi localizes to the nucleus (Cox et al., 2000), whereas Aub is cytoplasmic and associates with the perinuclear nuage (Harris and Macdonald, 2001). Vret protein expressed from a UASp-vret-myc transgene appeared cytoplasmic (see Fig. S4A-D in the supplementary material). To identify the cellular compartment in which Vret, Piwi and Aub might interact, we performed subcellular fractionations. In these experiments, the cytosolic and nuclear fractions of ovarian lysates were separated by differential centrifugation (Fig. 5F). In vret heterozygous extracts, Vret appeared in the cytosolic fraction, marked by Orb, whereas Piwi and Aub were found predominantly in the nuclear fraction, marked by Fibrillarin, and at lower levels in the cytoplasm (Fig. 5F), where it is most likely to interact with Vret. Piwi protein is thought to translocate to the nucleus once it is associated with piRNAs (Saito et al., 2009). By fractionating vret mutant ovarian extracts we found that the nuclear fraction of Piwi is affected more strongly than the cytoplasmic fraction (Fig. 5F). Since Vret is cytoplasmic, these findings suggest that Vret association with Piwi might facilitate the translocation of Piwi to the nucleus.

**Piwi stabilization is regulated uniquely in the gonad**

To determine whether Vret has a general role in Piwi translation or stability or it is specifically required for Piwi protein stability in the gonad, we ectopically expressed Vret and Piwi in the dorsal domain of the Drosophila wing disc, where neither is normally expressed (see Fig. S5A-H11032 in the supplementary material). In this heterologous tissue, Piwi protein, expressed as a UASp-HA-piwi transgene, was stable in the absence of Vret (see Fig. S5C-C/H11033 in the supplementary material). UASp-vret-myc transgenic expression alone was unable to induce Piwi expression, supporting the notion that piwi is not regulated by Vret transcriptionally or translationally (see Fig. S5B-B/H11630 in the supplementary material). Furthermore, the expression of a UASp-vret-myc transgene together with UASp-HA-piwi did not result in an increase of Piwi levels (see Fig. S5D-D/H11630 in the supplementary material). These results contrast with the loss of Piwi protein in the absence of Vret in the gonad, arguing that somatic and germline cells of the gonad employ a unique surveillance pathway regulating Piwi protein stability.

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**Fig. 4. vret is required for Piwi and Aub protein accumulation.** (A-D;) vret<sup>148-60</sup> germline and somatic mutant clones were generated at L3 and marked by the absence of GFP. (A-B;) Germline and somatic Piwi protein expression is dramatically reduced in vret mutant clones. (B-B;) An egg chamber with all follicle cells mutant for vret shows a dramatic reduction in somatic Piwi expression (outlined). (C-C;) Aub germline expression is severely affected in vret germline clones, whereas AGO3 expression (D-D;) is unchanged. GFP and the proteins tested are shown in separate channels for clarity. (E) Protein levels of Vret, Piwi, Aub, AGO3, Armi and Vasa are shown for wild-type (w<sup>1118</sup>), vret<sup>148-60/TM6</sup> and vret<sup>148-60</sup> mutant ovaries. α-Tubulin was used as loading control.
Primary piRNA production relies on Vret

Our data show that Vret is required for Piwi and Aub stabilization, as well as transposon control, suggesting a possible role for Vret in piRNA regulation. To determine which aspect of the piRNA pathway Vret affects, we cloned and sequenced 19-29 nt small RNAs from vret heterozygous and homozygous ovaries, and normalized libraries to the number of gene-derived, antisense-mapping endo-siRNAs as previously described (Brennecke et al., 2007) (see Materials and methods). To account for degraded RNA contamination, when possible, we analyzed small RNAs mapping antisense to active transposons, which would probably be derived from an active processing mechanism (Malone et al., 2009). We found that small RNAs in the piRNA range (23-29 nt) were dramatically diminished (Fig. 6A). In contrast to piRNAs, we found that overall levels of siRNAs (20-22 nt) were increased. This change can, however, be almost entirely attributed to a striking increase in MDG1-derived siRNAs (from 0.6% to 44.2% of total siRNAs in vret heterozygotes compared with mutants, respectively) (Fig. 6A). The specificity of MDG1 suggests that the increase in MDG1-derived siRNAs is a product of MDG1 de-repression in the absence of piRNA silencing, rather than a more direct effect of Vret on the endo-siRNA pathway.

As piRNA clusters are the primary source of transposon-targeting piRNAs, we analyzed changes in piRNAs mapping uniquely to the genome, ensuring that they were in fact derived from the corresponding cluster (Fig. 6B,C). We found that piRNAs from germline (42AB and Cluster 3) and soma (flam and traffic jam) as well as clusters expressed in both tissues (Cluster 2) were dramatically reduced in the absence of vret (Fig. 6B,C).

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we measured the 5’ complementarity of piRNAs matching individual transposons, with an expected 10 nt overlap due to slicer cleavage, as previously reported (Brennecke et al., 2008). Focusing on the Batumi and Roo transposons, we observed that primary piRNAs were preferentially lost in vret mutants, almost exclusively leaving 10 nt offset ‘ping-pong’ pairs in vret mutant ovaries (Fig. 6F,G); this is similar to observations in armi and piwi mutants, and in contrast to aub mutants, which affect ‘ping-pong’ amplification (Fig. 6F) (Malone et al., 2009; Olivieri et al., 2010). Together, these results indicate that Vret plays an essential role upstream of Piwi, and possibly Aub, in the primary piRNA pathway. Additionally, piRNA loss is most likely to underlie the transposon silencing defects observed in vret mutants.

**Vret does not affect piRNA cluster transcription**

To determine whether piRNA cluster transcription was affected in vret mutants, we analyzed the steady-state RNA levels of *Drosophila* piRNA clusters by qPCR. We focused on the unistrand-transcribed, somatic *flam* cluster and the dual-strand-transcribed, germline 42AB cluster (see Fig. S6 in the supplementary material). Ovaries mutant for *rhino* (*rhi*), which is required for cluster 42AB transcription (Klattenhoff et al., 2009), and for *flam*, in which *flam* transcript is undetected (Brennecke et al., 2007), were used as controls (see Fig. S6C,D in the supplementary material). In contrast to these controls, vret mutants showed no change in transcription from both the 42AB and *flam* clusters compared with heterozygotes (see Fig. S6A,B in the supplementary material), suggesting that Vret does not affect piRNA cluster transcription.

**DISCUSSION**

We identified a novel protein with critical roles in oocyte polarity, germline and soma differentiation, survival and transposon control. Vret, a Tudor-domain containing protein, associates with Piwi proteins in the cytoplasm of *Drosophila* ovarian cells and regulates their stability, as well as Piwi nuclear localization and localization of Aub to nuage. In the absence of Vret, piRNAs are dramatically reduced and transposons mobilized. By ordering the function of
Vret within the network of the piRNA-transposon-based system, we conclude that Vret functions in primary piRNA biogenesis at the stage of primary piRNA loading onto Piwi and Aub complexes.

Loss of Vret in the soma or germline has strikingly different morphological consequences. Our molecular analysis, however, suggests the same underlying cause for these defects: a failure to produce biologically active piRNAs. Morphologically, the vret germline phenotype resembles that of mutants defective in germline piRNA biogenesis, such as aub, spnE and krimper (Gillespie and Berg, 1995; Lim and Kai, 2007; Wilson et al., 1996). In these mutants, transposon mobilization activates a DNA damage checkpoint that leads to defects in transport and translation of maternal RNAs necessary for oocyte polarity and embryonic patterning (Chen et al., 2007; Ghabrial and Schupbach, 1999; Klattenhoff et al., 2007; Lim and Kai, 2007; Navarro et al., 2009). Interestingly, lack of vret in the soma resembles the piwi mutant phenotype, in which GSCs fail to differentiate as a consequence of somatic cell death, an event presumably associated with transposon misregulation. Thus, loss of vret in the germline and gonadal soma resembles loss of both Piwi and Aub. This, together with our findings that Vret associates with Piwi and Aub in ovarian extracts and affects the stability of both, strongly suggests that Vret regulates both proteins in a similar fashion.

Surprisingly, Vret is not required for piRNA ‘ping-pong’ amplification per se, suggesting that Vret might selectively interact with Aub and Piwi bound to primary piRNAs and not to those engaged in ‘ping-pong’. In this scenario, it would be possible for maternally deposited Aub to initiate the ‘ping-pong’ cycle with AGO3, even in the absence of Vret (Brennecke et al., 2008). As some Aub protein remains in vret mutant ovariates, an active pool of Vret-independent Aub could maintain ‘ping-pong’ activity throughout the adult ovary. Therefore, we propose that a ‘ping-pong’-independent pool of Aub within the cytoplasm depends upon primary piRNA loading, downstream of Vret function. It would be interesting to examine whether piRNAs associated with the Vret-dependent complex can, at any level, contribute to ‘ping-pong’, or whether Aub-bound primary piRNAs are functionally or enzymatically distinct from those involved in the piRNA amplification cycle.

In contrast to Aub, only a small subset of Piwi-bound piRNAs showed a 10 nt overlap with those bound to AGO3. Indeed, Piwi is genetically dispensable for ‘ping-pong’ and might be only marginally involved in ‘ping-pong’, if at all (Brennecke et al., 2007; Li et al., 2009). As Piwi slicer activity does not appear to be required for Piwi function (Saito et al., 2009), it seems most plausible that Piwi would act as a recipient, and not as an ‘active’ component of ‘ping-pong’ amplification. Regardless, the majority of Piwi-bound primary piRNAs act independently of ‘ping-pong’ and depend upon Vret for stability.

Our ectopic expression experiment suggests that Piwi is not ‘intrinsically unstable’, but becomes unstable in the gonad in the absence of Vret. Furthermore, Vret is not required for Piwi or Aub transcription or translation. Vret, therefore, could either coordinate the process of biogenesis and loading of primary piRNAs into Piwi and Aub complexes or be involved in stabilizing the mature RISC (RNA-induced silencing complex). Armi, a putative helicase, and Zucchini (Zuc), a member of the phospholipase D (PLD) family of phosphodiesterases, act like Vret in the soma and germline; they specifically affect Piwi protein stability and primary piRNA levels leaving the ‘ping-pong’ cycle intact (Haase et al., 2010; Malone et al., 2009; Olivieri et al., 2010; Pane et al., 2007; Saito et al., 2010).

Unlike Vret, the levels of unprocessed precursor RNA from flam are increased in zuc mutants implicating Zuc in piRNA cluster transcript processing. We therefore favor the hypothesis that Vret, possibly together with Armi, is an essential component of Piwi and Aub RISC complexes. Vret is one of many Tudor domain proteins in Drosophila that affects piRNA biogenesis and contains conserved residues that are known to be required for binding of sDMAs found in Piwi proteins (Sionov et al., 2010). When mutated, each of these genes displays a rather distinct phenotype. Krimper and SpnE regulate transposon levels in the germline whereas fs(1)Yb is soma-specific. Vret is, at this point, the only Tudor domain protein known to be required in both tissues, suggesting a conserved and global role for this gene in piRNA regulation. It remains to be determined whether the mammalian Tudor homolog could fulfill a similar function.

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Competing interests statement
The authors declare no competing financial interests.

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